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Cloning Sequencing and Structural Manipulation of the Enterotoxin D and E genes from Staphylococcus aureus.

Annual Summary Report

John J. Iandolo

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The regulation of the enterotoxin D (sed) has been investigated. Transcription in S. aureus initiates from a single promoter, but in E. coli 3 promoter sites were mapped. Activation of the staphylococcal promoter is dependent upon the product of the agr locus. In addition, another regulatory locus is operational and appears to be dependent on spacing of the end of the mRNA from the translational start codon.

Deletion analysis of the exfoliative toxin A gene has been initiated. A 72 amino acid C-terminal peptide was found to have exfoliative activity. However in vitro deletions of the gene to produce truncated toxin molecules missing portions of the C-terminus were transcriptionally inactive. Present evidence suggests that the 3' end of the Eta mRNA is important for message stability. A new regulatory locus that affects lipase production has been identified. Mutations in this region of the genome reduce lipase expression more than 90%. The locus has been cloned and efforts to identify regulatory gene products are underway.

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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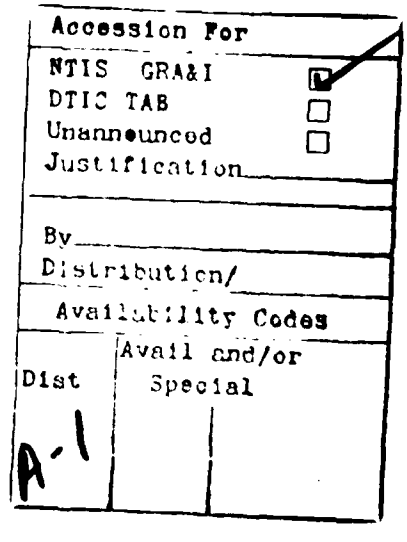

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During the last year of the contract we continued to make progress in achieving the major goals of the original proposal. We have 1) analyzed the regulation of the expression of the sed gene, 2) begun analysis of the transcriptional regulation and the active site of the exfoliative toxin A and B genes and 3) we have developed a quantitative assay for lipase and identified a second site trans-active regulator of lipase activity.

ENTEROTOXIN D

Localization of the sed transcription start site. The transcription start site of the sed gene was localized by S1 nuclease mapping. The results revealed the presence of a single protected fragment when using RNA isolated from S. aureus KSI1410 (Fig. 2, lane 2). The size of this fragment, determined by comparison to the simultaneously run sequencing ladder of the sed upstream region, maps the transcription start site to nucleotide (nt) -266 (Fig. 1). Just upstream from this site is a six base sequence that conforms perfectly (TATAAT, 6 of 6 matches) to the consensus -10 sequence for Bacillus sp. and E. coli promoters. Although a sequence similar to the consensus -35 sequence (TTGACA) is also present, it contains mismatches (TAGAGG, 3 out of 6 matches) and is separated from the -10 sequence by only 14 nucleotides rather than the consensus separation of 17 to 18 nucleotides.

RNA from the E. coli clone, JM109 (pIB488), gave rise to three protected fragments (Fig. 2, lane 3). These fragments identify transcription start sites at nt -266, as observed when using S. aureus RNA, and also at nts -109 and -200 (Fig. 1). Sequences upstream from both of these sites that are similar to the consensus -10 and -35 sequences were also present (Fig. 1).

In order to determine if the transcription start sites localized at nts -109 and -200 were involved in sed expression in S. aureus, a promoter deletion construct was made by digesting the sed plasmid, pIB489, with the restriction enzymes, Fnu4HI (cleavage site shown in Figure 1) and HindIII (cleaves 3' to the sed gene). This digestion isolated the sed structural gene and the promoters at nts -109 and -200 on a single DNA fragment while omitting the promoter at nt -266. This fragment was then ligated into the shuttle vector, pLI50 to form pIB476. After introduction of pIB476 into S. aureus RN4220 and E. coli JM109, the strains were assayed for SED. As shown in Figure 3a (lane 2), SED was produced by the S. aureus RN4220 strain containing the sed plasmid, pIB586 (contains all promoter sequences determined from Figure 2). However, when the promoter at nt -266 was absent, as in S. aureus RN4220 (PIB476), sed expression was reduced by 94% (Fig. 3a, lane 4). When the wild-type and deleted promoter constructs were placed in the E. coli JM109 background, both expressed the sed gene,

as predicted by the S1 nuclease protection analysis (Fig. 2, lanes 2 and 3). The expression of sed observed with PIB476 in E. coli was probably the result of transcription initiation from the promoters at nts -109 and -200. Therefore, considering the data in Figures 2 and 3, transcription of the sed gene in S. aureus appears to be initiated exclusively at nt -266.

Regulation of sed by agr/exp. The S. aureus regulatory gene, agr (also known as exp), has been shown to control the expression of a number of extracellular protein genes. The effects of the agr/exp gene product are at the transcriptional level and can either increase or decrease expression. To determine how agr/exp influences the expression of sed, PIB586 was transformed into the agr⁻/exp⁻ S. aureus strain, ISP546. The relative amount of SED made by this genetic construct (compared to that made by strain RN4220) was determined by analyzing extracellular proteins by Western blotting and densitometric scanning of the blot. These data show that the expression of sed was reduced 82% in ISP546 (Fig. 3, lane 5) compared to expression of sed in RN4220 (Fig. 3, lane 3). The reduction in the amount of SED in ISP546 is also reflected in the amount of sed-specific RNA present in this strain compared to strain RN4220 (a 91% reduction, see figure 5).

To determine if the inverted repeat located between nt -146 and nt -107 of the sed leader sequence (Fig. 1) was involved in the regulation of sed, a

plasmid was constructed with this region (190 bp) deleted. The plasmid (pIB479) was transformed into S. aureus strain RN4220 and assayed for SED production. The amount of SED secreted (Fig. 4, lane 3) was reduced 49% compared to that secreted by the wild-type construct, PIB586 in the same genetic background (Fig. 4, lane 2).

The inverted repeat deletion construct (PIB479) was also transformed into ISP546 and assayed for SED production. SED made by this strain (data not shown) was reduced by 95% compared to RN4220 (PIB586). When compared to the wild-type gene in the agr⁻/exp⁻ background, SED production was reduced an additional 48%. The additive effect of these two mutations (the agr/exp and the inverted repeat deletion mutations) indicate that the inverted repeat is not involved in the regulation of sed by Agr/Exp.

To determine if the deletion in PIB479 affected the transcription and/or stability of the sed transcript, the amount of sed-specific RNA transcribed from PIB586 and PIB479 was compared. A 38% (Fig. 5) reduction in the amount of the sed transcript was observed when compared to that produced by the strain containing PIB586. This reduction in the amount of sed transcript was reflected in the amount of SED made. In addition, sed RNA levels in the agr⁻/exp⁻ strain containing PIB479 were 50% of that expressed in the wild-type strain containing PIB586 (Fig. 5). These data lend further

support for the hypothesis that the inverted repeat is not involved in the regulation of sed by Agr/Exp.

Message stabilizing activity of the 5' untranslated portion of the sed transcript was assessed. The 190 bp deletion in PIB479 was replaced by a heterologous DNA (i.e. lacking an inverted repeat sequence) of the same size. This plasmid, designated pIB483, was transformed into strain RN4220 and assayed for SED production. As shown in figure 4 (lane 4), this strain produced approximately the same amount of SED (determined by densitometry) as that made by strain RN4220 containing PIB586 (Fig. 4, lane 2). This implies that the reduced Sed level produced by strain RN4220 containing pIB479 is due to the length of the 5' untranslated portion of the sed transcript and not to the inverted repeat sequence itself.

Since the sequence of the sed inverted repeat is similar to the inverted repeats of the promoter regions of iron regulated genes in Gram-negative organisms (data not shown), we suspected that the sed gene might also be regulated by iron. Furthermore, if sed is an iron-regulated gene, the effects of an inverted repeat mutation would only be detected under low iron conditions. Therefore, the production of SED by KSI1410 in deferrated medium was analyzed. The amount of SED made in deferrated TSB was the same as that made in untreated TSB or in deferrated TSB supplemented with

iron (data not shown). However, other proteins that cross-reacted with the anti-SED antiserum were influenced by the iron concentration. We have not had time to pursue this observation.

DNA/protein interactions. To determine if the inverted repeat could serve as a site for protein binding, the cytoplasmic proteins from strain RN450 were isolated (this strain was selected since sed was expressed as efficiently as in the wild-type strain). These proteins were passed through a DNA-cellulose column which only bound those that had the capacity to bind double-stranded DNA. The bound proteins were then serially eluted by washing the column with buffer containing increasing NaCl concentrations.

To detect DNA binding activity the fractions were analyzed using a mobility shift assay. This technique is based on the principle that a DNA/protein complex migrates slower in a 4% polyacrylamide gel than an unbound DNA molecule. As shown in Figure 6 (lanes 2 and 7), the protein fractions eluted with 0.25 M and 0.6 M NaCl contained proteins that bound to the 153 bp DNA probe that contained the inverted repeat. In addition, a 1000-fold excess of an unlabeled DNA fragment containing the inverted repeat (Fig. 6, lanes 5 and 10) competed for binding with the labeled DNA. However, a similar unlabeled DNA fragment corresponding to the inverted repeat deletion competed for binding as efficiently as that containing the

inverted repeat (Fig. 6, lane 6 and 11). These data indicate that the DNA binding proteins are not specific for the inverted repeat sequence.

Overall, these results indicate that the sed gene is regulated by the agr locus of S. aureus. However, other data suggest that additional modes of transcriptional control may affect the expression of Sed. The finding of three promoter sites utilized in E. coli suggest that some type of temporal control may exist, at least in this background. A large inverted repeat sequence which does not serve as a binding site for Agr proteins or transcripts was also investigated. This sequence which is present in several other extracellular proteins, but not in typical cytoplasmic proteins examined may also indicate that other forms of regulation may exist. However, we were unable to find any proteins that bound specifically to this region of the sed gene nor were we able to titrate specific DNA binding proteins with a genetic construct that contained only the inverted repeat region of sed. However, presently unknown temporal and/or nutritional considerations may serve to regulate expression of such binding proteins. Preliminary data suggest that the spacing of the transcription start site from the translation start has a significantly affects the stability of the messenger RNA in an as yet undetermined manner. However, investigation of these hypotheses is yet to be initiated and would depend upon continued funding.

EXFOLIATIVE TOXINS

Prerequisite to studies of the mode of action of ETA and ETB, we attempted to determine the sites of biological activity within the molecules. We hypothesized that the C-terminal region of the proteins was important for biological activity. This is a region where 81% of the amino acids of ETA and ETB (17 of 21 residues at positions 201-221) are identical (35). Moreover, preliminary evidence in our laboratory showed that a cyanogen bromide derived peptide of ETA containing the 72 C-terminal amino acid residues which includes the region of greatest similarity, caused exfoliation in neonatal mice (42) (unpublished data). The goal of this research was to generate a panel of nested deletions in eta and to test the deleted clones for production of biologically active ETA.

Deletions of pJJ825, the wild type plasmid containing the entire Eta gene, were made using Exonuclease III and Mung Bean Nuclease. Three plasmids with deletions of 203, 278, and 591 bp were selected for further experimentation. The inserts were cloned into pUC18 for gram negatives and pLI50 for gram positives and transformed into E. coli JM109 and E. coli SG23006 Lon$^-$ and into S. aureus RN4220.

Using computer modeling, we determined that the molecular weights of the ETA proteins produced from pJJ830, pJJ834, and pJJ835 should be

13,887; 29,458; and 32,222, respectively. These data were obtained by alignment of the junction sequences of the eta inserts to the sequence of PUC18. This enabled us to determine the extent of translational read-through before encountering a termination signal in pUC18. The molecular weight of ETA produced from pJJ825 is 26,950. We failed to detect any ETA on a Western blot from the deleted constructs in E. coli JM109. These results indicated that transcription and/or translation were being affected. If this were true, it would appear that the 3' end of eta is important for these processes.

One hypothesis regarding the absence of ETA production from the JM109 plasmid containing strains, was that the proteins produced from the deleted clones were unstable. Perhaps unstable, deleted proteins were rapidly degraded by E. coli proteases. This hypothesis was rejected by the results obtained from a Western blot analysis of ETA produced in the Lon strain SG23006. As was true for the deleted constructs in JM109, only the isolate containing the wild type plasmid produced ETA. ETA was not produced from the deleted clones in SG23006. Apparently, the Lon protease of E. coli was not affecting ETA.

The results of the Western blots from E. coli strains containing the various constructs, were similar to those from S. aureus. This was true in

spite of the fact that in the E. coli strains, transcription was initiated from the lac Z promoter and in the S.aureus strains, transcription signals from eta were utilized. ETA was not produced by KSI863 or KSI864, those strains containing deletions in eta of 591 and 278 bp, respectively. KSI865, the isolate containing a 203 bp deletion in eta, produced a small quantity of ETA in comparison to KSI866, the wild type S. aureus clone. These data suggest that the 3' region of the eta gene is important for message and/or protein stability. The junction between the eta inserts and pLI50 was not determined, so we did not know the expected sizes of the proteins as we did for the E. coli pUC18 constructs.

RNA was isolated from our S. aureus clones and analyzed by a slot blot to estimate the stabilities of the mRNA transcripts. The results showed that no mRNA was produced from KSI863 lending support to the hypothesis that the 3' region of eta which was deleted in this clone is important for message stability. The same conclusion may also be implied for KSI864 although this isolate produced a small amount of mRNA. pJJ864 contains an additional 313 bp in the 3' region of eta which are not present in pJJ863. Perhaps the nucleotides in this area are important for increased mRNA stability. KSI865, the isolate containing only a 203 bp deletion, produced a significant amount of mRNA, almost equal to that of the wild type, KSI866, but ETA production

was only a fraction of the wild type level. Again, the finding suggests that the 3' region of eta is important for increased message stability. When 278 bp are deleted from the 3' area, transcription seems to be greatly affected, but it is only slightly affected when 203 bp are eliminated. The specific role of these additional 75 nucleotides is unknown; the sequence of this region reveals no obvious secondary structure which may be involved in transcription or translation.

Because KSI865 produced a significant quantity of mRNA, we expected it to produce a significant quantity of protein. This was not the case. Only a small amount of ETA was detectable. The eta insert in pJJ865 contains only 10 amino acid residues fewer than full-length ETA. Nevertheless, our data suggest that the region coding for these 10 amino acids is necessary for complete and effective translation. How the 3' region of eta is involved in message and/or protein stability is unknown.

The results of the neonatal mouse assay were not surprising in light of the Western blot data. If we were unable to detect ETA from the deleted clones on an SDS-PAGE gel, then it was unlikely to expect a positive Nikolsky sign in mice injected with toxin samples from these clones. Only the mouse injected with whole cells from KSI866 showed flaking and peeling of the epidermis. Even the E. coli strains containing pJJ825 (KSI825 and

KSI858) did not show exfoliation. It is possible that the E. coli strains do not produce enough toxin to be detected biologically. Another possibility may be that the E. coli inoculum does not survive well in the mouse due to host defenses. Confirmation of either explanation necessitates further research.

From mRNA analysis, we expected to see exfoliation in the mouse injected with KSI865 cells. A significant amount of message was produced by this clone (although we do not know its molecular size). However, as this strain produced little protein, the mouse result was not surprising.

Further research will be required to determine the exact site of biological activity within the ETA molecule. It will be necessary to discover more precisely the role of the 3' region of eta in transcription and translation. Northern blot analysis would be useful for determining the sizes of mRNA produced. Such data would reveal whether the messages produced from KSI864 and KSI865 were intact or degraded. This information would be important in addressing the question of mRNA stability.

Site-directed mutagenesis experiments may pinpoint one or a few nucleotides which are essential for activity. Hydropathicity data show similar hydrophobic regions in ETA and ETB. One of these regions is in the C-terminal area of the proteins. Such similarity between the two proteins suggests that the three-dimensional folding of the molecules is similar.

Presumably, the proteins, therefore, can present similar sites to an appropriate target. Preliminary crystallography results have been published for ETA, but the level of resolution is insufficient to determine folding of the molecule. High resolution X-ray crystallography studies are currently in progress (M. Sax, personal communication) to determine the folded structure of ETA. This information is needed to discover which sites in the toxin are available to interact with a proposed target molecule.

LIPASE

We have developed a simple and sensitive quantitative assay for measurement of lipase activity. Most published assays require complicated and expensive chromatographic analysis to determine the amount of fatty acid hydrolysed from triglycerides. However, this simple assay can be used for lipase determinations in agar plates or in spectrophotometric analysis which improves precision. The assay is based on the fact that triglycerides are insoluble in water solutions, but β-glycerides are soluble. Lipase cleaves end fatty acids from triglycerides liberating β-glyceride. We therefore, emulsified 0.25% tributyrin in a solution of 0.1% carboxymethylcellulose (added to stabilize the emulsion) by sonication for 7 - 10 minutes at 50w. This suspension was used to analyze lipase activity in solutions of lipase to standardize the reaction kinetics and from filter sterilized culture

supernatants. Reduction of optical density at 450nm was measured. The reaction produced a linear decrease in O.D. whose slope was indicative of lipase activity. Reaction velocity also varied linearly with dilution of enzyme concentration. The reaction was specific in that no activity was observed with lipase negative strains that produced other lipolytic enzymes (phospholipases C and D). In addition, sufficient sensitivity exists in the assay to measure activity differences in transcriptionally regulated lipase mutant strains.

Use of this assay allowed characterization of a trans-active lipase regulatory element. We identified this novel regulatory locus by transposon mutagenesis using Tn551. An insert designated Ω1058 which maps to the right of thy in segment A on the standard staphylococcal chromosome map was found to significantly depress the expression of geh (lipase gene) which maps in segment E. Because these two loci are roughly 180° degrees apart on the circular chromosome, it is almost certain that Ω1058 has inactivated a trans-active positive regulatory element. We have been able to transduce the mutation into other Lip$^+$ strains and reproduce the mutant phenotype. We have determined that this mutation in the original mutant strain and in the transductant strains simultaneously down-regulates hly and hlb, the α and β toxin genes and seb (the enterotoxin B gene), but up-regulates coa

(the coagulase gene). This effect is substantially different from the effect of the Agr mutation which has no effect on geh, down-regulates hly, hlb, and seb but up-regulates coa. Further, restriction mapping of the chromosome of S. aureus has shown that agr and Ω1058 map in different locations. Therefore this appears to be a separate regulatory locus. We have cloned the Tn551 insertion and used this clone to reprobe the wild type chromosome. The wild type sequences have also been cloned onto the cosmid pLAFR3 on a 25 kbp DNA fragment. Present efforts are designed to subclone and identify the specific regulatory locus on this fragment.

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5. Gordon, J.A. and J.J. Iandolo. 1990. Stability of the mRNA encoding exfoliative toxin A is dependent on 3' sequence integrity. In preparation for submission to Infect. and Immun.

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THESES

1. Gill, S.R. Genetic analysis of extracellular protein synthesis in Staphylococcus aureus. PhD dissertation. 1989.

2. Gordon, J.A. Deletion analysis of the exfoliative A gene of Staphylococcus aureus. MS thesis. 1990.

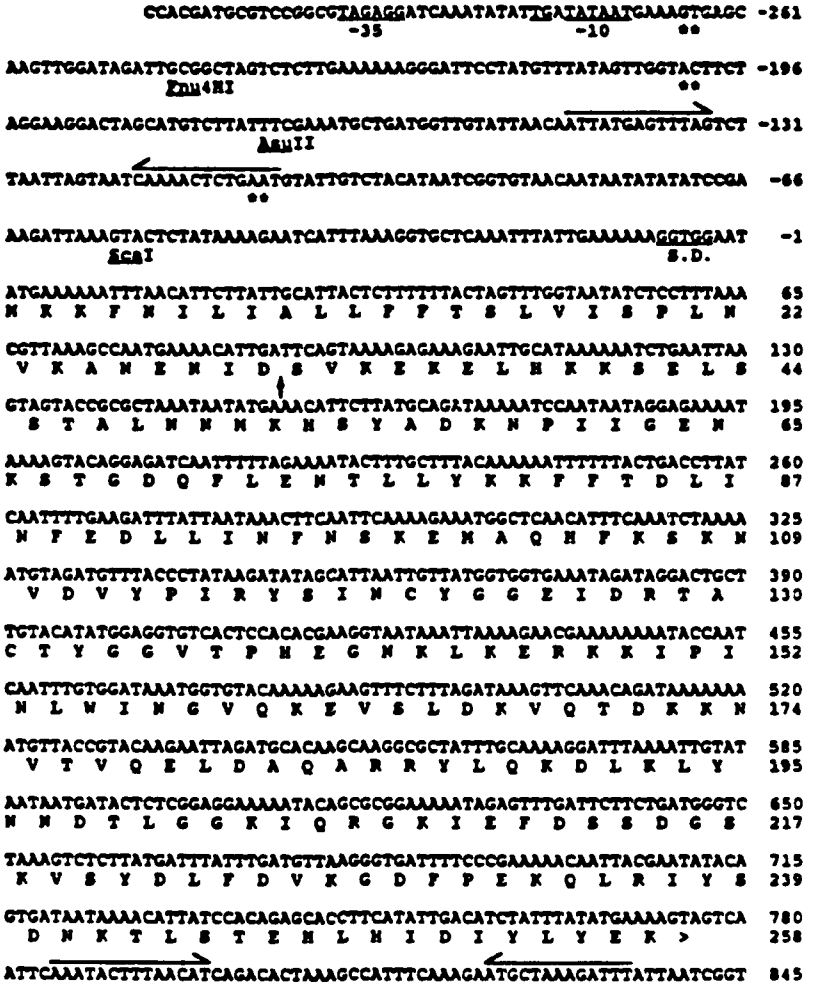

Fig. 1. Sequence analysis of the entD gene. Shown is the nucleotide sequence of the entD open reading frame which encodes a 258 amino acid protein. The deduced amino acid sequence is also presented. The leader peptide cleavage site is indicated by a vertical arrow. The horizontal arrows near the 3' end of the entD gene indicate a possible transcription termination signal.

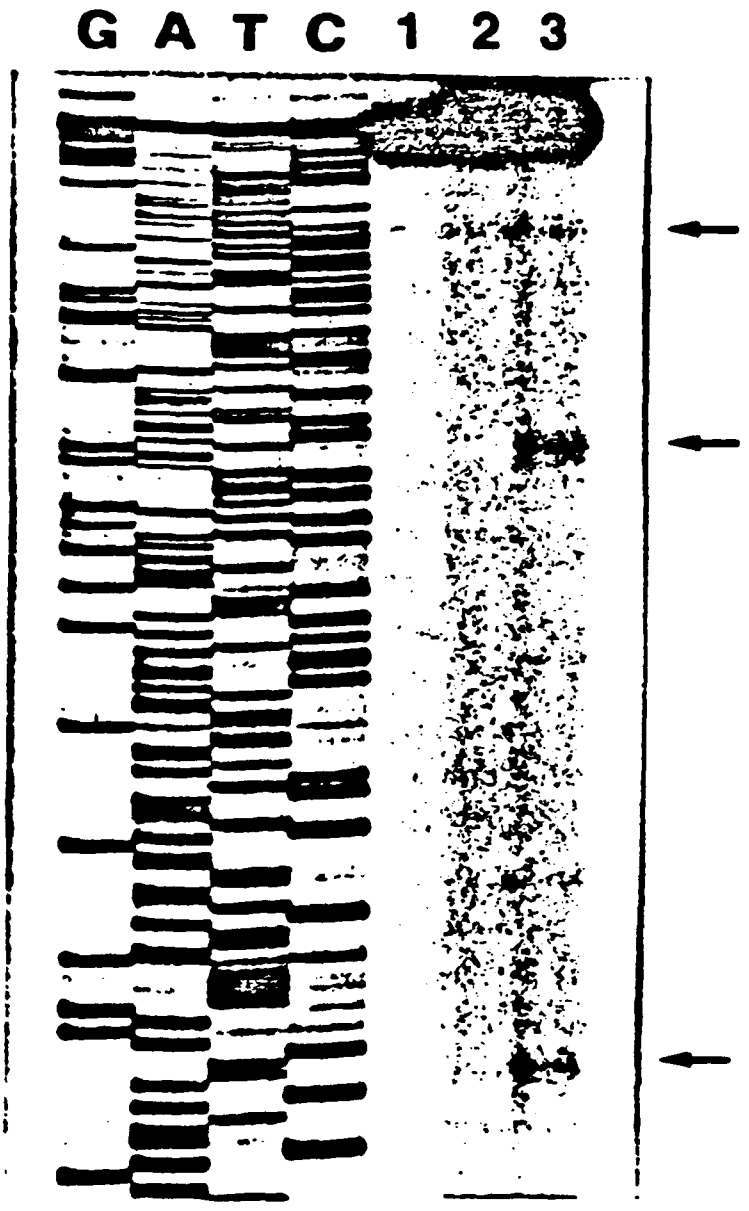

Fig. 2. Analysis of the transcription start site of the entD gene. Total RNA was isolated from S. aureus KSI1410 (lane 2) and E. coli KSI1454 (lane 3) and used to hybridize to ^{32}P labeled fragments containing the upstream sequences of entD. A hybridization was also carried out using no protecting RNA (lane 1). The samples were digested with S1 nuclease and seperated on a sequencing gel. Protected fragments were detected by autoradiography.

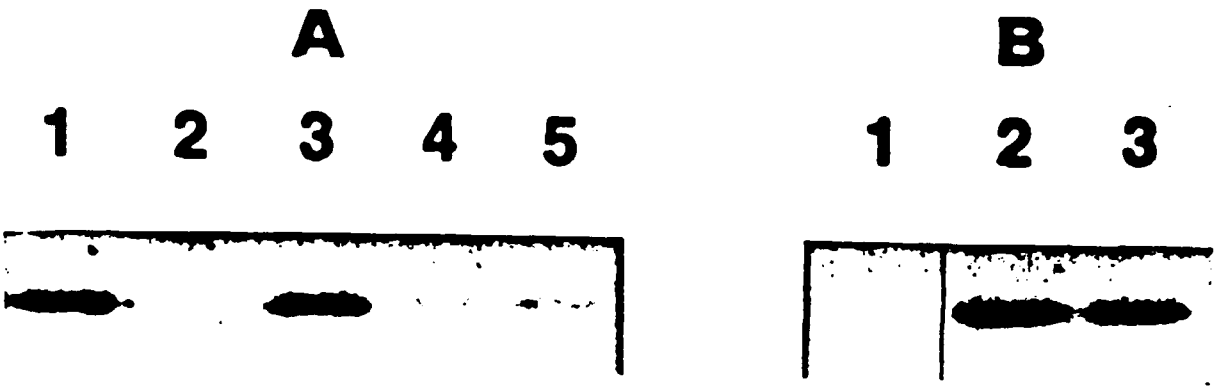

Fig. 3 . Western immunoblot analysis of the expression of entD in S. aureus and E. coli strains containing entD plasmid constructs. a) Equal amounts of extracellular proteins from 18 h cultures of the S. aureus strains. Lanes: 1, partially purified SED (100 μgs); 2, RN4220 (pLI50); 3, RN4220 (pIB586); 4, RN4220 (pIB476); and 5, ISP546 (pIB586). b) Total proteins from 4 h cultures of the E. coli strains. Lanes: 1, JM109 (pLI50); 2, JM109 (pIB586); and 3, JM109 (pIB476). SED was detected using anti-SED antisera and ^{125}I labeled protein A.

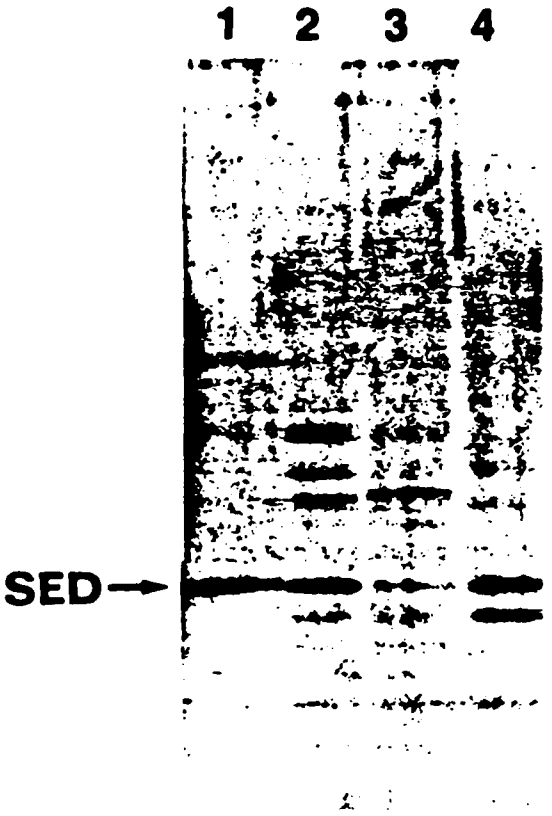

Fig. 4. Western immunoblot analysis of *entD* mutants in *S. aureus*. Equal amounts of extracellular proteins from 18-h cultures were seperated in a 15% polyacrylamide gel and blotted to nitrocellulose. Lanes: 1, partially purified SED (100 μg); 2, RN4220 (pIB586); 3, RN4220 (pIB479); and 4, RN4220 (pIB483). SED was detected using anti-SED antisera and ^{125}I labeled protein A.

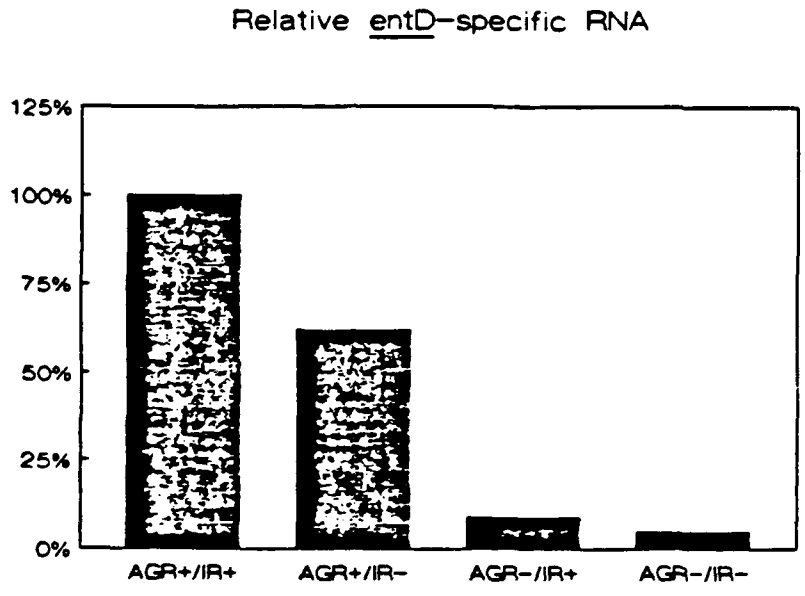

Fig. 5. The relative amount of entD-specific RNA made in mutants of S. aureus. Equal amounts of total RNA from RN4220 (pIB586) (AGR+/IR+), RN4220 (pIB479) (AGR+/IR-), ISP546 (pIB586) (AGR-/IR+), and ISP546 (pIB479) (AGR-/IR-) were added to GeneScreen Plus in a slot blot apparatus and hybridized to the ^{32}P-labeled entD containing DNA insert in pIB479. The amount of probe hybridizing was detected by autoradiography and quantitated by densitometric scanning of the autoradiogram.

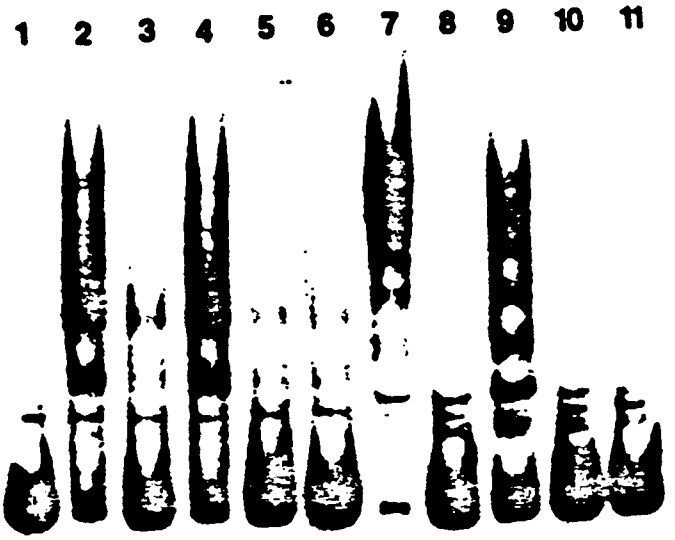

Fig. 6. Gel-retardation assay of the 0.25 M NaCl (lanes 2-6) and 0.6 M NaCl (lanes 7-11) protein fractions eluted from a DNA-cellulose column (see materials and methods). DNA fragments containing the inverted repeat were end-labeled with ^{32}P-dATP and incubated in the presence of a 1000 fold molar excess of unlabeled DNA fragments containing the inverted repeat (lanes 3 and 8); a 100 fold molar excess of unlabeled DNA fragments containing the inverted repeat (lanes 4 and 9); a 1000 fold molar excess of unlabeled 1.3 Kbp DNA insert from pIB586 (contains inverted repeat, lanes 5 and 10); and a 1000 fold molar excess of unlabeled 1.1 Kbp DNA insert from pIB479 (has inverted repeat region deleted, lanes 6 and 11). Lanes 2 and 7 contain DNA samples incubated in the absence of competitor DNA and lane 1 contains a DNA sample incubated in the absence of protein extract. The DNA/ protein mixtures were seperated in a 4% non-denaturing polyacrylamide gel and visualized by autoradiography.

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